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(54) Title: LHRH-ANTAGONISTS

(57) Abstract

This invention consists of two aspects: 1) the method of design and synthesis of LHRH antagonists; 2) the products thereafter obtained by using the above method. Taking (NAc-D2Nal[1], DpClPhe[2], D3Pal[3], Ser[4], Tyr[5], DArg[6], Leu[7], Arg[3], Pro[9], DAla[10])NH2 as the parent compound, a series of new analogs expressed as (NAc-D2Nal[1], AA[2], AA[3], Ser[4], AA[5], AA[6], Leu[7], AA[3], Pro[9], DAla[10])NH2 are obtained by fine modification of both lipophilic area and alkaline area of the molecule of the parent compound. In this way, the high antiovulatory activity of the parent compound can be maintained and the histamine releasing activity can be reduced to the level so as to meet the clinical requirement.

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LHRH-ANTAGONISTS

Specification

The products and their application of method for design and synthesis of luteinizing hormone releasing hormone antagonists

The present invetion relates to novel peptides and their derivatives having exact chemical structure. The invention is also directed to the methods of their preparations and applications. Hypothalamic luteinizing hormone releasing hormone (LHRH) acts on the anterior pituitary gland to stimulate the secretion of luteinizing hormone (LH) and follicular stimulating hormone (FSH). The antagonistic analog of LHRH acts on anterior pituitary rapidly, lasts a long duration, can be safely and reversibly used for contraception or selectively suppression of gonadotropin secretion. For such kind of application, LHRH antagonists are superior to agonists. Up to now, there are more than two thousands of LHRH analogs have been designed and synthesized, among which "Nal-Arg" analog showed fairly high antifertility activity. However, "Nal-Arg" analog showed also very strong histamine-releasing activity (HRA). It caused transient edema of the face and extremities in rats when administrated at a dosage as high as 50-100 times of therapeutic dose. The result of clinical trial demonstrated histamine-related systemic effects. Other LHRH antagonists containing DArg^6 or DLys^6 showed similar side effects, their ED_{50} for HRA were below

1 µg/ml. The present invention provides new LHRH antagonists which have very high antiovulatory activity (AOA) and very low histamine-releasing activity HRA) and negligible side effects.
The contents and examples of this invention are as follows:

The design methodology of this invention is based on the topological similarity between the molecule of parent compound [NAc-D2Nal^1, DpClPhe^2, D3Pal^3, Ser^4, Tyr^5, DArg^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2 (II) and a neoropeptide, Substance P, which features the modification of both alkaline and lipophilic area in t molecular of the parent compound to give new antagonis having both high AOA and low HRA. The term "modification" hereof is adjusting or substitution of the amid acids in he area of Tyr^5-DArg^6-Arg^8 in C-terminus and the aromatic acids in N-terminus of (II More specifically, the design is introduction of suita alkaline group and substitutions of unnatural amino asids in position 2, 3, 5, 6, 8 of (II).

The following are also the methods and examples of this invention.

1. Substitution of D3pal which is a aromatic amino acid having suitabele basicity for DArg^6 in (II) t obtain analog (III): [NAc-D2Nal^1, DpClPhe^2, D3Pal^3 Ser$_4$, Tyr$_5$, D3Pal$_6$, Leu$_7$, Arg$_8$, Pro$_9$, DAla$_{10}$]NH_2

2. Substitution of Arg^5 for Tyr^5 in (III) to obtain (IV): [NAc-D2Nal^1, DpClPhe^2, D3Pal^3, Ser^4, Arg^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2

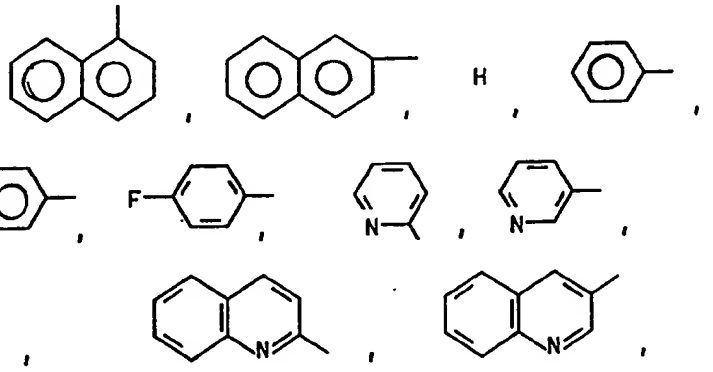
3. Substitution of Dphe^3 or its derivatives DXCH$_2$Phe for D3pal^3 in (IV) to obtain (V): [NAc-D2Nal^1, DpClPhe^2, DPhe^3, Ser^4, Arg^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH$_2$ or its (DXCH$_2$Phe^3) analogs.

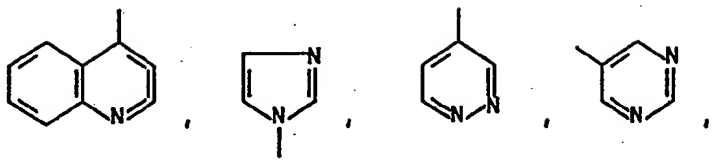
4. Substitutions of Dphe^3 or its derivates for D3pal^3 in (III) to obtain (V'): [NAc-D2Nal^1, DpClPhe^2, DPhe^3, Ser^4, Tyr^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH$_2$ or its (DXCH$_2$Phe^3) analogs.

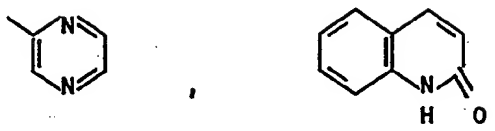
A series of new LHRH antagonists of the formula [NAc-D2Nal^1, AA^2, AA^3, Ser^4, AA^5, AA^6, Leu^7, AA^8, Pro^9 DAla^{10}]NH$_2$ have been synthesized, where AA are natura or unnatural amino acids which are expressed as D- or L-ArAla. More specifically herein,

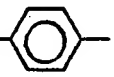
AA^2 = D-pClPhe, D-ArAla, DPhe, Ar-Ala, DXCH$_2$Phe;
AA^3 = D3Pal, Ar-Ala, D-ArAla, DPhe, D-XCH$_2$Phe;
AA^5 = Arg, DMap, Pip, Tyr, Pal, Mop, Tep, Map, Phe, Eap, Pap, Bap, DMop;
AA^6 = D3Pal, D-Ar-Ala, D-XCH^{2}Phe;
AA^8 = Pip, Mop, Tep, Map, Eap, Pap, Bap, Arg;

in which

Ar = 





and XCH_2—  ,

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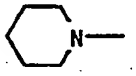
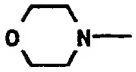
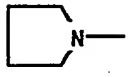
X=  ,  ,  ,

and R'_2N-, RR'N-

in which

R' = CH_3-, CH_3CH_2-, C_3H_7-, C_4H_9-, H-;

R = CH_3-, CH_3CH_2-, C_3H_7-, C_4H_9-, H-;

The LHRH antagonists obtained by using the above described method, as a kind of peptide medicine, can be used to treat the disorder of reproductive endocrine system, such as edometriosis, precocious puberty of childeren and to treat prostate cancer and

breast cancer as well as used as male or female contraceptives for birth control, or used in the diagnosis and treatment of intertility, etc. Such peptide medicine can be prepared as normal injection injectable capsules or other formulatations for real application.

Further description of this inventation is as follows:

In the natural course of histamine releasing in the body, neuropeptide substance P plays a very important role, its ED_{50} for HRA is 5-15 μM. The chemical structure of SP is [Arg^1, Pro^2, Lys^3, Pro^4, Gln^5, Gln^6 Phe^7, Phe^8, Gly^9, Leu^{10}, Met^{11}]NH_2. The study on the relationship between its structure and HRA showed that Arg^1-Pro^2-Lys^3 in the N-terminus in the molecule of SP is essential for its HRA because deletion of these three amino acids from the molecule entirely abolished its HRA. By contrast, deletion of one two or three amino acids in C-terminus remained HRA as high as one fourth as HRA o itself. Further deletion of Phe^8 and Phe^7, HRA reduced 4 % and 0,57 % of those of SP. Further deletion of $Gln^{5.6}$ did not cause significant change of HRA. The above data implies that the liphophilic area around $phe^{7.8}$ determines the value of HRA, this area involves in the binding of molecule with the receptor of mast cell.

As mentioned previously, ($D2Nal^1$, $DArg^6$) analogs of LHRH showed very high HRA, its molecular structure has topological similarity with SP: $DArg^6$-Leu^7-Arg^8 in the former appears to be corresponding to Arg^1-Pro^2-Lys^3 in the latter, both consist of a pair of strongly basic amino acid residues between which only one neutral amino acid residue is present, i. e. both [$D2Nal^1$,DArg analog of LHRH and SP contains two

strongly basic amino acid residues which are in 1,3 posititon relation ship. On the other hand, a clutter of aromatic amino acid residues in the former is considered to be corresponding to $Phe^{7,8}$ area in SP in terms of determination of he magnitude of HRA.

The design of this invention consists of two aspects: one is modifying Tyr^5-$DArg^6$-Arg^8 area in C-terminus, the other is fine adjusting the aromatic acids after optimizing the modificatin of the alkalious area in C-terminus. [NAc-$D2Nal^1$, $DpClPhe^2$, $D3Pal^3$, Ser^4, Tyr^5, $DArg^6$, Leu^7, Arg^8, Pro^9, $DAla^{10}$]NH_2 (II) is used as parent compund, which showed AOA 100 % at 0,5 µg in coin oil, 57 % at 0,25 µg.

First, $DArg^6$ in (II) could be replaced by weekly basic or neutral aromatic acids, such as D3Pal, D6Qal, tetrahydrotryptophan, methyl tryptophan. [NAc-$D2Nal^1$, $DpClPhe^2$, $D3Pal^{3.6.}$, $DAla^{10}$]LHRH(III) was optained when $D3Pal^6$ was substituted for $DArg^6$ in (II). (III) showed AOA 100 % at 3 µg, 83 % at 1 µg (in corn oil), and its ED_{50} for HRA was 9.8 µg/ml, much better than that of "Nal-Arg" analog ED_{50} for HRA was less than 1 µg/ml. It seems that the basicity of the whole molecule should equal to or closed to that of a pair of arginine in order to obtain high AOA. Because position 5 , like position 6, does not involve in the receptor binding, a wide variety of amino acid including arginine can be inserted in position 5. A series of new analogs were designed. For example, substitution of Arg^5 for Tyr^5 in (III) gave [NAc-$DNal^1$, $DpClPhe^2$, $D3Pal^{3.6.}$, Arg^5, $DAla^{10}$] LHRH (IV). Both (IV) and (II) contained two arginines, but the distance between two arginines in (IV), whose geometric relationship became 1, 4, i. e. there were two other amino acids between these two

arginine, was larger than that in (II). Therefore, HRA would be reduced and, on the other hand, because of th presence of two arginine, AOA should not be lower than that of (II). The bioassay result of (IV) showed that ED_{50} for HRA was 3,5 µg/ml, while AOA was 60 % at 0,12 µg (corn oil), 85 % at 0,25 µg, 100 % at 0,5 µg. This was the first time for LHRH antagonists to achieve ED_{50} for AOA which was equal or less than 0,125 µg.

Therefore further design was based on the structure of (IV).

There are four alkalions residues, $D3Pal^{3,6}$ and $Arg^{5,8}$ in the molecule (IV), while (II) contains only three alkalinous. Therefore, it is resonable to replace one D3Pal by a neutral amino acid; on the other hand, (IV) showed very strong hydrophilicity and reducing the hydrophilicity by the substitution of a hydrophobic amino acid for DPal in (IV) would be beneficial to the retention of the drug in the body and then to the extension of the effective duration. A new series of analogs were then designed by substitution for $D3Pal^{3}$. (V) showed 100 % of AOA at 1 µg (in saline), equal to that of parent compund (IV), while HRA redued by a half: ED_{50} for HRA was 7,4 µg/ml.
Further substitution of $DPhe^{2}$ for $DClPhe^{2}$ reduced the lipophilicity of this area in the molecule and reduced HRA.

Arg^{5}-$D3Pal^{6}$-Leu^{7}-Arg^{8} in the C-terminus of (IV) seems to play a major role in triggering histamine releasing. D3Pal combines aromacity, basicity and hydrophilicity in one molecule, it is also stero-acceptable in LHRH antagonists for receptor binding. Similiarly, design of new series of unnatural amino acids prosessing the same

character as D3Pal may lead to better LHRH antagonists that (IV) or (V).

Modification of natural, lipophilic, aromatic amino acid e. g. phenylalanine, for example, by means of the method described below in The Syntheis of Novel Unnatural Amino Acids, lead to a series of novel amino acids which combine aromacity, hydrophilicity and basicity in one melecule and can expressed the as formula: $R_1R_2NCH_2C_6H_4CH_2CH(NH)CO_2H$ (VI), were R_1 and R_2 may be the same or may differ each other, may be chain-like or cyclic, may also contain hetero-atom. With the R_1 and R_2 change, a series of amino acid can be obtained, which show systemically changed basicity, hydrophilicity and stero-character. Introduction of those amino acids in position 5, 6, 8 of (IV) have given three series of new antagonists of LHRH. The bioassay results showed that each series gave at least one new antagonist showing 100 % AOA at 1 µg, similar to that of (IV), while HRA was significantly reduced. An example was (VII): [NAc-D2Nal^1, DpClphe^2, D3Pal^3, Ser^4, Mop^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]-NH_2, which showed 100 % of AOA at 1 µg, ED_{50} 14,7 µg/ml for HRA, appeared better than (V). When substitution of (VI) for Arg^8 in (IV), the extent of HRA decreae was positively correlated to the length of R in (VI), so ED_{50} for HRA could be higher than 200 µg/ml, such kind of compounds can be easily dissolved in aqueous solution and expected to be utilized clinically without formulation problems. The results demonstrated that Arg^8 or Lys^8 was not essential for highly potent LHRH antagonists. Suitable alkaline center in position 8 can ensure high AOA, meanwhile activity inducing mast cell to release histamine was remarkablely reduced when the basic enter mentioned above possesing significant stero-hinder.

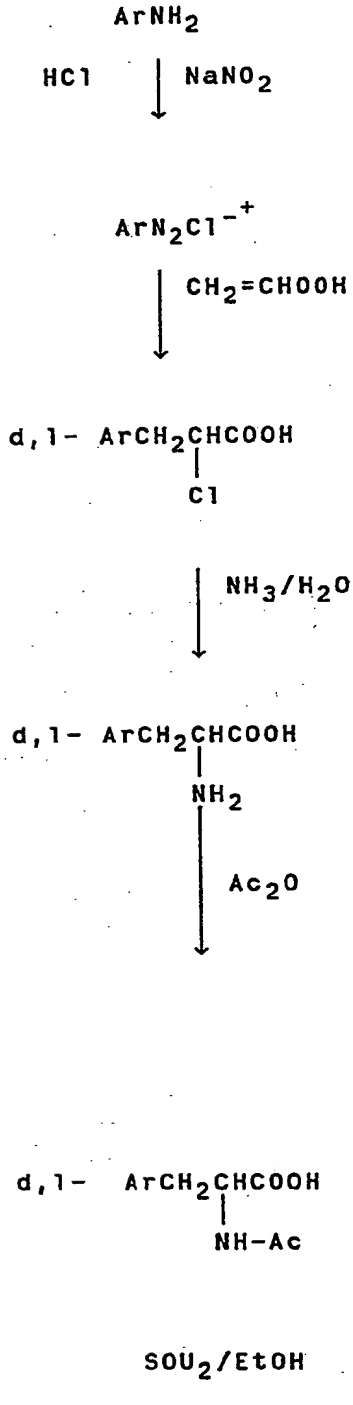
This invention combining the modification in both N- and C-terminus lead to better LHRH antagonists.

The process of synthesis are illustrated as follows:

1. The synthesis of Novel Unnatural Amino Acids

Over 60 series and nonseries, D- or L-amino acids are designed and synthesized through the four synthetic routes outlined in the schema below. The structure of these unnatural amino acids are shown with the general structural formulas listed in the same schema. Some of these amino acids have alkalinity, hydrophilicity and aromaticity respectively, while the others have them all together in the molecule.

Path 1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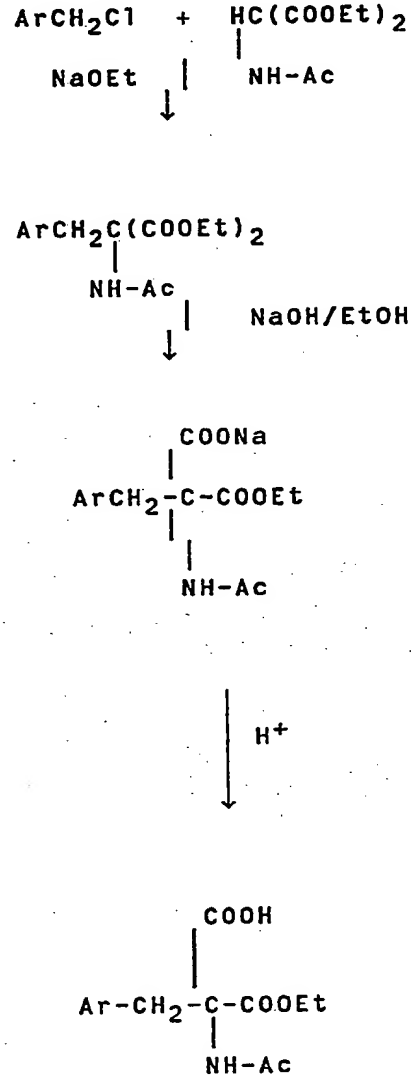
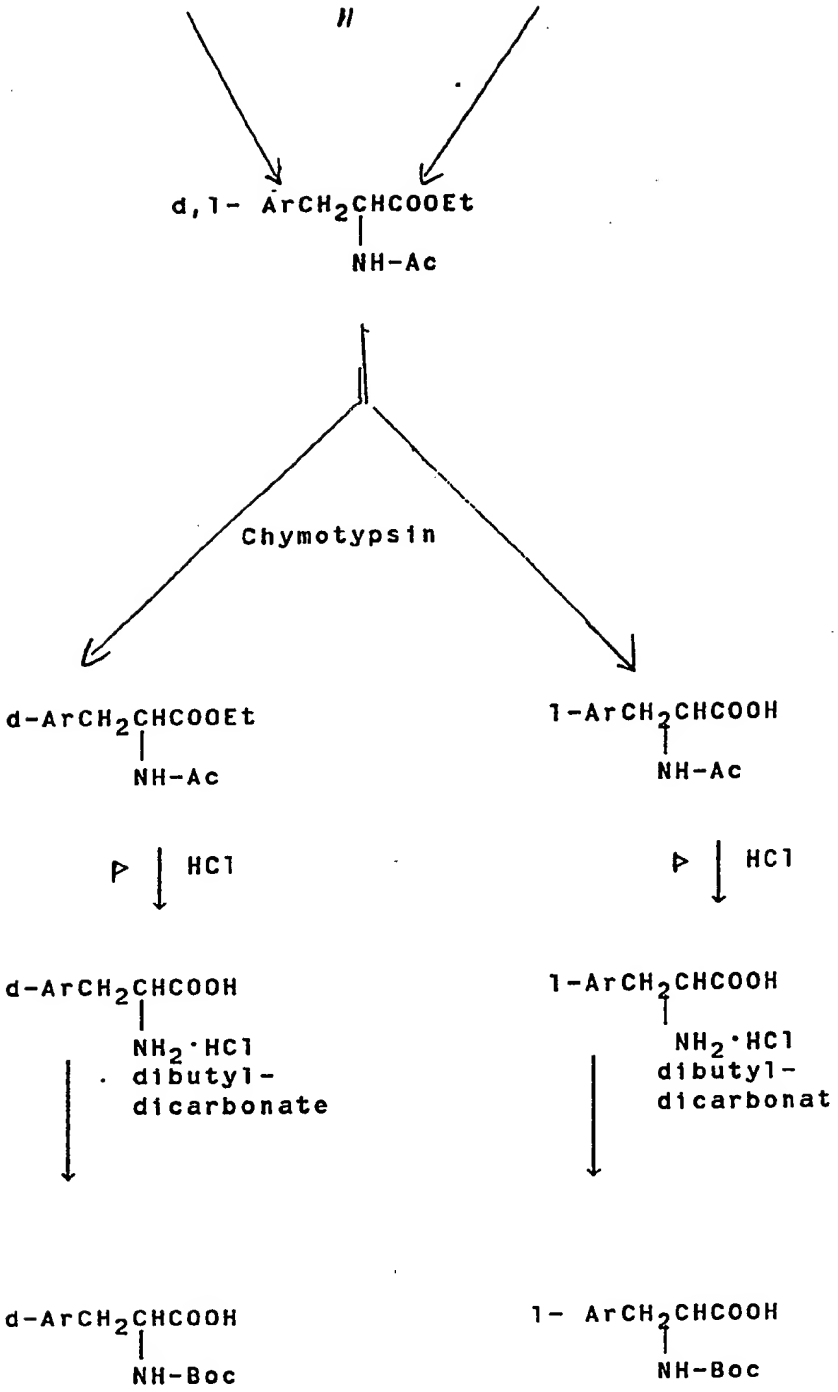
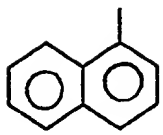
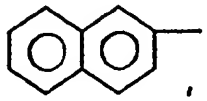
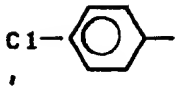
HCl
NaNO2
ArN2Cl-+
CH2=CHOOH
d,l- ArCH2CHCOOH
Cl
NH3/H2O
d,l- ArCH2CHCOOH
NH2
Ac2O
d,l- ArCH2CHCOOH
NH-Ac
SOU2/EtOH

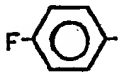
Path 2


ArCH2Cl + HC(COOEt)2
NaOEt
NH-Ac
ArCH2C(COOEt)2
NH-Ac
NaOH/EtOH
COONa
ArCH2-C-COOEt
NH-Ac
H+
COOH
Ar-CH2-C-COOEt
NH-Ac

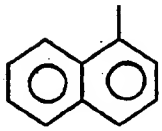
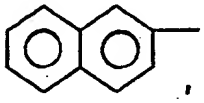
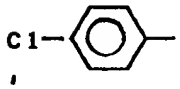

II
d,l- ArCH2CHCOOEt
NH-Ac
Chymotypsin
d-ArCH2CHCOOEt
NH-Ac
l-ArCH2CHCOOH
NH-Ac
HCl
HCl
d-ArCH2CHCOOH
NH2·HCl
dibutyl-
dicarbonate
l-ArCH2CHCOOH
NH2·HCl
dibutyl-
dicarbonat
d-ArCH2CHCOOH
NH-Boc
l- ArCH2CHCOOH
NH-Boc

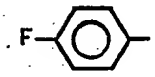
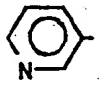
Path I D or L-Ar-CH2-CH(NH-Boc)-COOH wherein

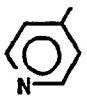
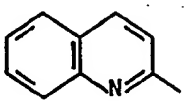
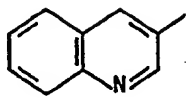
Ar =  ,  ,  ,

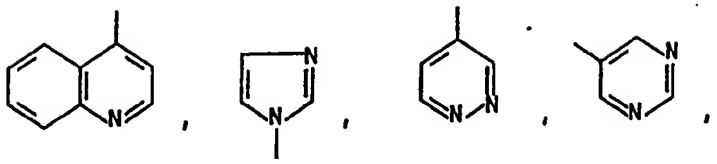


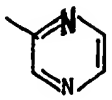
Path II D or L-ArCH2-CH(NH-Boc)-COOH wherein

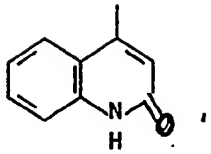
Ar =  ,  ,  ,

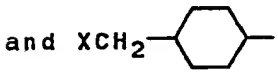
  

 ,  ,  ,

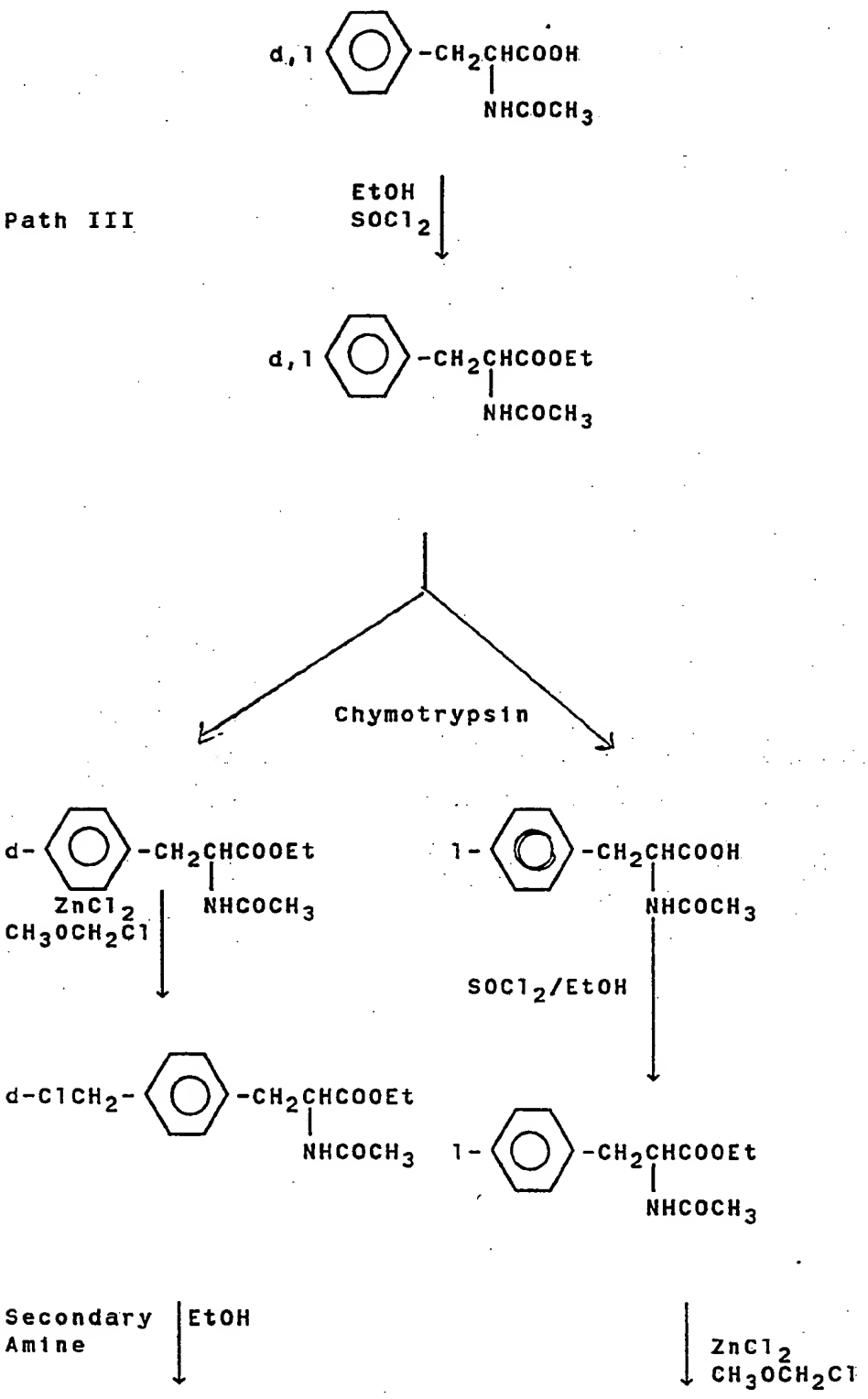


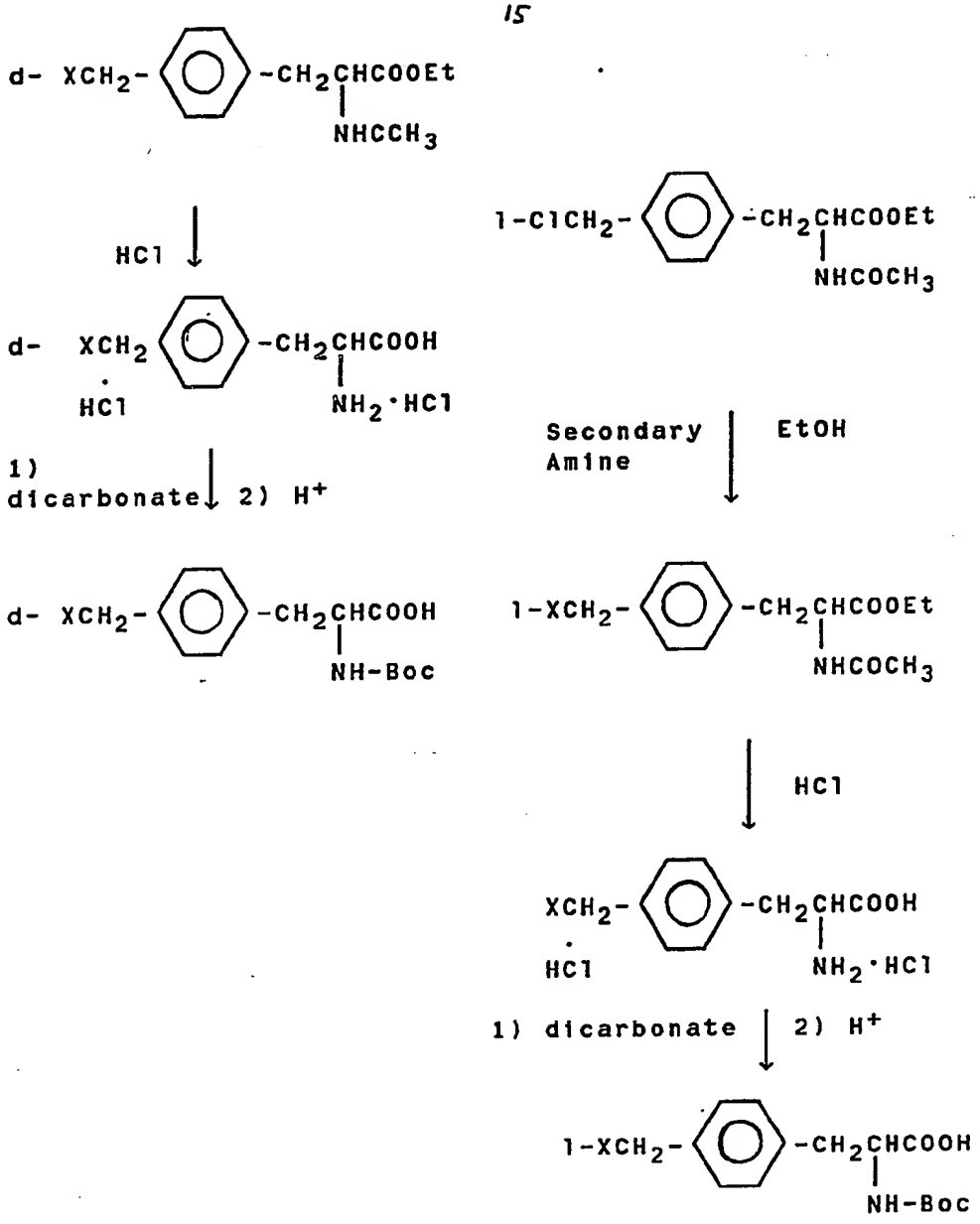




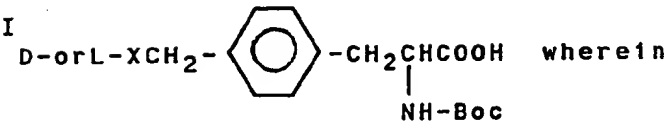
and 

X= 

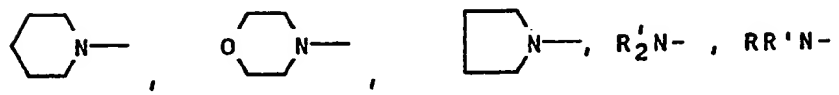

d,l -CH2CHCOOH
NHCOCH3
EtOH
SOCl2
Path III
d,l -CH2CHCOOEt
NHCOCH3
Chymotrypsin
d- -CH2CHCOOEt
NHCOCH3
ZnCl2
CH3OCH2Cl
l- -CH2CHCOOH
NHCOCH3
SOCl2/EtOH
d-ClCH2- -CH2CHCOOEt
NHCOCH3
l- -CH2CHCOOEt
NHCOCH3
Secondary Amine
EtOH
ZnCl2
CH3OCH2Cl



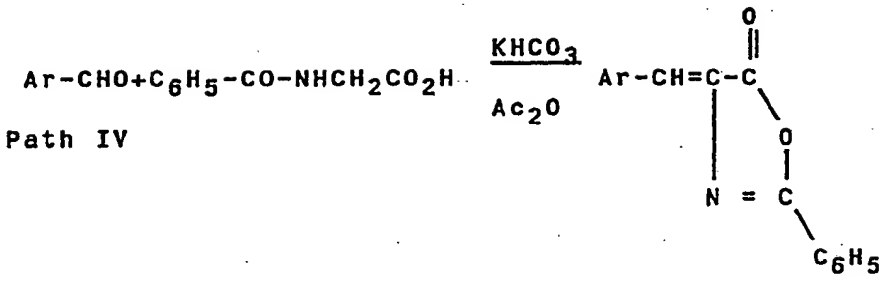
Path III

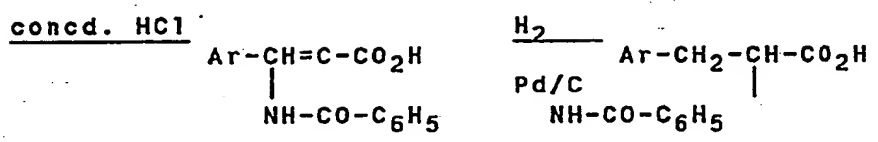


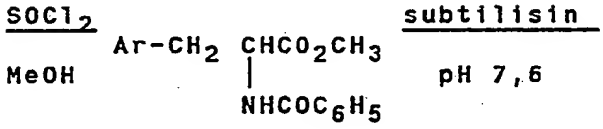
wherein

X= 

16


Ar-CHO+C6H5-CO-NHCH2CO2H
KHCO3
Ac2O
Ar-CH=C-C
O
O
N = C
C6H5
Path IV

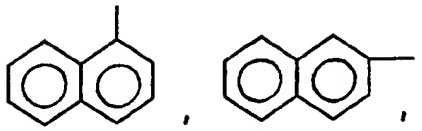

concd. HCl
Ar-CH=C-CO2H
NH-CO-C6H5
H2
Pd/C
Ar-CH2-CH-CO2H
NH-CO-C6H5

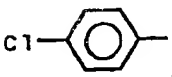
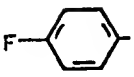
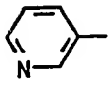

SOCl2
MeOH
Ar-CH2 CHCO2CH3
NHCOC6H5
subtilisin
pH 7,6

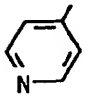
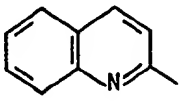
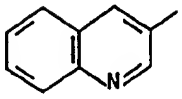

Ar-CH2CHCO2CH3
NHCOC6H5
D-isomer
6NHCl
+
Ar-CH2CHCO2H
NHCOC6H5
L-isomer
6NHCl

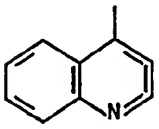
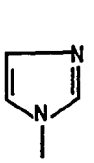
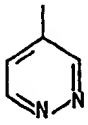
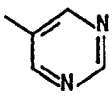

Ar-CH2CH-CO-OH
NH2
(D-amino acid)
Ar-CH2CH-CO-OH
NH2
(L-amino acid)

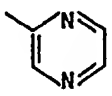
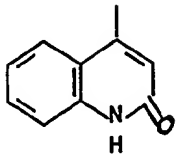
Path IV D or L Ar-CH_2-CH(-NH_2)-COOH, wherein

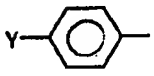
Ar =  , , ,

 ,  ,  ,  ,

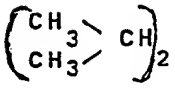
 ,  ,  ,

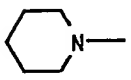
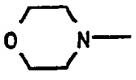
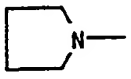
 ,  ,  ,  ,

 ,  ,

 ,

Y = $(CH_3)_2N$-, $(CH_3CH_2)_2N$-, $(CH_3CH_2CH_2)_2N$-,

 N-, $(CH^3CH^2CH^2CH^2)^2N$-

 ,  ,  ,

2. Synthesis of Peptide

The synthesis begins from the C-terminus of the peptide on benzhydrylamine hydrochloride resin (BHA resin) utilizing the method of solid-phase peptide synthesis introduced by Merrifield. It is a three-step process including anchor, coupling and cleavage. Dichloromethane (DCM) is the major solvent used for washing between each step of reaction while isopropanol alcohol (IPA) and N,N-dimethylformamide (DMF) are also used when it is necessary. Catalyzed by excessive dicyclohexylcarbodiimide (DCC), coupling reaction is carried out, while adequate amount of 1-hydroxybenzotriazole (HOBT) is added. The degree of the coupling reaction is monitored with Kaises ninhydrin method. The second coupling reaction would be carried out if it gives a positive result in Kaises test. The peptide chain is cleaved from the resin using anhydrous hydrogen fluoride (HF) in the presence of anisole after the completion of all reactions necessary on the resin, all of the temporary protecting group are deprotected at the same time. After washed by ethyl acetate or ether crude products of LHRH antagonists are obtained by aqueous acetic acid extraction followed by lyophilization. The yield is over 50 %.

3. Purification of Peptide

(1) The peptide is purified by gel permeation chromatography or silica partition chromatography through a column as high as 60 - 100 cm with the aid of UV/TLC monitoring. The LHRH antastits

purified once are obtained after lyophilizing the major fractions. The yield is 50 - 90 % and the purity can be over 90 %.

(2) The peptide then is further purified on Waters high performance liquid chromatography (HPLC) instrument using reverse phase C18 column (7,8 x 300 mm) (μ-Bondapak 84176). The yield of this step is 20-50 % while the purity is no less than 99 %.

4. Purity Analysis of Peptide

(1) TLC analysis
It is carried out on a plastic sheet coated by silica gel 60 F254 of 5 - 10 cm height. They all shows a single spot when developed in five different solvent systems.

(2) HPLC analysis
They all shows a single peak when eluted with two kinds of solvent system, respectively, utilizing Waters HPLC instrument on a analytic column (μ-Bondapak 27324) when monitored by UV 210. The sample size is 10 - 200 μg.

5. Amino Acid Analysis of Peptide:

According to the PICO-TAG method developed by Waters Company, 50 μg of sample which have been dried under vacuum over 2 hours is weighed accurately on a 10^{-5}g scale balance. After dissolved in water, 10 μg of aliquot is added to a reaction tube in which 1:1 hydrochloride acid (containing 1 % phenol) was added according to the manual.

The reaction lasts 22-24 hours at 105°C in a sealed container which had been filled with nitrogen and pumped to vacuum to remove the oxygen in reaction tube. Phenol isothiocyanate is added to derive the amino group after evaporating of excessive hydrochloride acid. Then it was analyzed with the HPLC-instrument equipped with PICO-TAG amino acid analytical column and monitored by UV254. The content of each amino acid and the relative mole ratio were calculated to give the amino acid composition of the sample based on the comparison of the integrated area of each amino acid to that of H-standard sample of Waters. The classical ion-exchange-ninhydrin derivation method (IEN) was also used as control which gave the same results. But it needed ten times more sample to get a satisfied result.

6. Evaluation of biological activity:

Corbin's rat antiovulation method is used. The healthy, adult, female SD rats (BW 200-250 g) are used in this experiment. All animals are maintained at 22-24 °C and on 14 h/10 h (light/dark) schedule. They are given standard food, and water ad libitum. The rats showing at least two consecutive 4-day estrous cycles in vaginal smear examination can be used in this experiment. The rats are given peptides (LHRH antagnists) at noon of proestrous with different dose in saline solution. The rats are sacrificed next day, their oviduct of two sides are examed under a dissecting microscope to determine the ovum number. The rats were divided into several groups according to the dosing, each group

consists of about 10 rats, and the control group in which the rats are given equal amount of saline consists of 9-10 rats. The antiovulatory activity (AOA) is shown in the following equation:

$$AOA = \frac{\text{number of unovulated rats}}{\text{Total number of treated rats}} \times 100\ \%$$

7. Evaluation of Histamine Releasing Activity:

(1). Histamine releasing test (HRT) in vitro:

The healthy, adult, male SD rats (BW 200-250 g) housing in the above same conditions are used in this experiment. After anesthetized by CO_2 the peritoneal cavity is washed with 50 ml of PIPES AC medium containing 20 units of heparin. Following centrifugation at 200xg for 8 min at 4 ^{o}C, cells are washed again and finally resuspended to a concentration of 8 to 24x10^5 total leucocyted/ml in PIPAS AC. This suspension contains approximately 5-10 % mast cells. Washed cells are used immediately after collection and are prewarmed for 5 min at 37 ^{o}C prior to pipetting 0,3 ml aliquots into polystyrene tubes containing 0,3 ml of diluted peptide. The mixtures are incubated for 15 min at 37 ^{o}C and the reaction stopped by centrifugation at 400 xg for 15 min at 4 ^{o}C. The supernatans are assayed for histamine content by manual fluorometric assay method after successively extraction with n-butanol and n-heptane. The histamine content can be obtained from the histamine standard curve (see below). The percentage of histamine release can be calculted from the following equation:

$$\text{Histamine release (\%)} = \frac{E-B}{C-B} \times 100\ \%$$

where E is the fluorometric reading of experimental sample, B is the fluorometric reading of samples with cells and buffer only, and C ist the fluorometric reading of "complete" (cells treated with $HClO_4$).

The standard curve can be obtained by plotting the OD values on a fluorometer at 350 nm/450 nm (activation/fluorescent) against the concentrations of serially diluted solution of accurately weighted histamine hydrochloride. The relative parameter r of the histamine standard curve can be 0.9998, and the lowest detectable concentration of histamine is 0,5 ng/ml.

The ED_{50} value of peptide can be gotten from the dose response curves obtained by plotting the histamine release versus the peptide concentration on semilogarithmic paper.

All peptide samples should be tested with mast cells from a minimum of 3 different rats.

(2). Cutaneous anphylactoid activity test (CAT):

The healthy, adult, female SD rats (BW 250 g) are used in this experiment. The rats are injected

intravenously with Evan's blue (1ml of 0,05 % solution). Immediately after that, the 0,05 ml of peptide solution (5, 0,5 and 0,05 µg/ml respectively) and saline (control) are injected intradermally into a shaved section on the back of the animals. 30 minutes after the injection, the rats are sacrificed and the dorsal skin was reflected. The diameters of the lesions are measurd in millimeters in two perpendicular directions with a vernier caliper. The diameter of control is usually less than 5,5 mm.

The amount of Evan's blue permeating into the skin from the blood vessel can be spectrophometrically measured, too. The skin corresponding to the lesion area is cut down and immersed in a mixture of acetone/saline (7:3, Vol/Vol) overnight. After centrifugation next day, the content of Evan's blue in the supernatant is measured with a spectrophotometer (UV-260) at 610 nm against reference solutin of acetone/saline (7:3). Each peptide were tested in a minimum of 3 different rats.

A variety of new LHRH antagnists were designed and systhesized by means of the method described above. In brief, the new structure of LHRH antagonists was obtained by single or multiple substitution of the various natural and unnatural amino acids listed in the previous paragraphs.

A part of examples of new LHRH antagonists obtained thereupon are illustrated in table 1

Table 1: A Part of Examples Related to This Invention

Analogue	AA 1	AA 2	AA 3	AA4	AA5	AA 6	AA7	AA8	AA9	AA 10
Parent	NAc-D2Nal	DpClPhe	D3Pal	Ser	Tyr	DArg	Leu	Arg	Pro	DAla-NH2
					Arg					
					Arg	DPhe		Pip		
					Arg	DMop		Pip		
					Arg	DPhe		Mop		
					Mop	D3Pal				
			DPip		Mop	DMop		Pip		
					Arg	D3Pal		Pap		
					Arg	D3Pal		Pip		
					DFPhe	D3Pal		Pap		
						DPip		Eap		
						DMap		Mop		
			DPhe		Arg	DMop		Map		
			DPClPhe			DPip		Map		
			DPhe		Arg	D3Pal				
					Eap			Mop		
					Tep	DMop		Pep		
					Tep	DMap		Mop		
					Tep	DEap				
					Tep	DBap				
					Tep	DPap				
					Tep	DTep				
		DpFPhe	D3Pal			DMop		Mop		
		DPhe			Mop	DMop		Eap		
						DPip		Pap		

Table 1: A Part of Examples Related to This Invention, Page 2

Analogue	AA 1	AA 2	AA 3	AA4	AA5	AA 6	AA7	AA8	AA9	AA 10
Parent	NAc-D2Nal	DpClPhe	D3Pal	Ser	Tyr	DArg	Leu	Arg	Pro	DAla-NH2
						DBap		Pip		
					Tep	DMop		Eap		
						DMop				
						DTep				
					Tep	DMop				
						DTep				
		DPClPhe								
		DPClPhe				DMop		Pip		
						DMop		Bap		
					Nop					
					Nop	D3Pal				
					Arg	DMop				
					Arg	DPip				
					Arg	DTep				
						D3Pal		Pip		
					Arg	D3Pal		Pip		
					Arg	D3Pal		Nop		
					Arg	D3Pal		Tep		
					Arg	D3Pal		Pap		
					Arg	DTep		Pip		
					Arg	DTep		Nop		
			DPhe		Arg	DTep		Tep		
			DFPhe		Arg	DTep		Nap		
			DFPhe		Arg	DTep		Eap		
			DFPhe		Arg	DTep		Pap		
			Nep		Tep	DPap		Bap		

The Applications of This Invention:

1. After finishing the preclinical pharmacology and toxicology study, we can apply these new LHRH antagonists which have high therapeutic effectiveness and low side effect in clinic so as to develop new medicine for treating the endometriosis and their disorder in reproductive endocrine system including precious puperty of children, prostate cancer and breast cancer. Since they suppress the secretion of gonadotropin through competing receptor with endogenous LHRH, and act rapidly reversibly and safely, they can be further developed as new type of contraceptives for male or female. Besides, they can be also used in treatment of infertility and for selectively and reversibly abolishing the function of pitutary gland in terms of secreting gonadotropin.

   Being a kind of peptide medicine, the LHRH antagonists desrcibed herein are unlikly to be administrated orally. But they can be easily made into lyophilized powder which are ready to dissolve in saline for injecting iv, sc or im.

   Morover, long-actin delivery systems, such as biodegradable, injectable capsules are studied. The capsules can be implanted subcutaneously by a special syringe and would be adsorbed by the tissue after release of all peptide contents and do not need to remove surgically. The long-acting delivery system is specially useful for long-term adminstration of LHRH analogues in clinic.

The following are the analyses results of the examples (taking three analogues IV, V, VII as typical examples):

(1) The Purity

Thin layer chromatography (TLC):

There is only a single spot in each of the chromatogram developed in five different solvent systems.

High performance liquid chromatography (HPLC):

There is only a single peak in each of the chromatogram eluted with two different sovent systems.

The values of Rf and retention time TR are shown in Table 2, also with reference of Figure 1-4.

Table 2: The chromatographic analysis results of LHRH antagonists

Analogs	HPLC TR1	HPLC TR2 (min)	TLC Rf1	TLC Rf2	TLC Rf3	TLC Rf4	TLC Rf5
IV	7.55	5.26	0.23	0.21	0.31	0.19	0.65
V	7.90	8.11	0,32	0.30	0.35	0.30	0.69
VII	16.19	9.58	0.17	0.08	0.16	0.40	0.12

Solution A + 80 % acetonitrile

Solvent System 2:

Solution A is 0.01M KH_2PO_4 aqueous solution (pH3)

Solution B is 20 % solution A + 80 % acetonitrile

TLC solution system:

1. nBuOH/EtOAC/HOAC/H_2O (5:5:1:1)
2. nBuOAC/nBuOH/HOAC/H_2O (2:8:2:3)
3. nBuOAC/HOAC/H_2O (4:1:5), up phase
4. nBuOH/HOAC/H_2O (4:1:2)
5. nBuOH/EtOAC/HOAC/H_2O (1:1:1:1)

(2) Amino acid analysis

The analysis are carried out according to the method of classical IEN and new PIco-Tag, the results are shown in Table 3 and Figure 5,6.

Table 3: The amino acid composition of LHRH antagonists

Ana-logs	Methods	Ser	Arg	Ala	Pro	Leu	Phe	Pal	pClPhe	Nal
IV	IEN	0,86	2.05	1.01	0.99	1.13		+	+	ND
	Pico-TAG	0,92	2.25	0.91	1.01	0,91		+	+	+
V	IEN	0.81	2.02	1.03	1.03	0.12	0.99	+	+	+
	Pico-TAG	0.68	2.26	0.93	1.29	1.04	1.00	+	+	+
VI	IEN	0.91	0.91	1.00	1.00	1.09		+	+	ND

ND: Not determined

(3) The bioassay results

The results of bioassays including antiovulatory activity at different doses and ED_{50} for histamine-realising activity in vitro are illustrated in table 4 in which 26 antagonists are listed as examples.

Table 4: Bioassay Results of New LHRH Antagonists based on Parent structure

		% AOA/µg					HRA (µg/ml)
	Substituted Amino Acids	0.125	0.25	0.5	1.0	2.0	ED_{50} ± SEM
1	Parent		50	75	100		3.5 ± 0.38
2	DPhe		29	60	100		7.4 ± 0.98
3	DPhe,DPhe				0		18.5 ± 7.00
4	DTyr, Lys				40		5.1 ± 2.15
5	D-Phe					60	35.0 ± 5.05
6	Map			29			24.8 ± 4.47
7	Eap			43			12.0 ± 0.50
8	Pap			0			9.6 ± 0.19
9	Bap			14			23.5 ± 5.78
10	D-Map			12,5			18,3 ± 2,38
11	Tep			14			36.8 ± 5.68
12	Pip	17	33	71	100		9.4 ± 1.63
13	Mop			25	100		14.7 ± 2.70
14	D-Map			14			19.5 ± 2.50
15	D-Eap			14			13.0 ± 1.00
16	D-Tep			71			22.5 ± 3.25
17	D-Pip			0	50	57	7.6 ± 2.48
18	D-Mop		33	67	100		> 11
19	Map			57	100		5.4 ± 1.22
20	Eap				29		56.9 ± 15.1
21	Pap				50	88	70.4 ± 26.8
22	Bap				0		> 235
23	Tep				100		6.6 ± 2.13
24	Pip				43		27.5 ± 2.50
25	Mop				71		52.5 ± 17.5
26	D-Map				0		28.0 ± 9.00

* The parent structure is: [NAc-D2Nal[1], DpClphe[2], D3Pal[3], Ser[4], Arg[5], D3Pal[6], Leu[7],Arg[8], Pro[9], DAla[10]]NH_2

As illustrated and described above, the LHRH antagonists designed and synthesized according to this invention shows very good properties. They are pure in TLC or HPLC analysis. They are pure in TLC or HPLC analysis. Their compositions are correct, i. e., the same is designed. Their antifertile activity is high: they can inhibit rat ovulation when injected s. c. at the dosage of 0.1 to 2.0 µg at the noon of proestrus. Their histamine related side effect is low: their ED_{50} for in vitro histamine releasing activity (the effective dose for rat mast cell to release 50 % of histamine) is ranged 5-300 µg/ml; the lession induced by them in the cutaneous anaphylactoid test in rats is as small as required in clinic. Their warer-solubility is very good, all bioasays are carrid our in saline solution, so they are easy to formulated for injection in clinic. They are also ready to formulated as long-acting delivery systems, among which injectable microcapsules are most convenient for long-term suppression of gonadotropin and gonadal hormone. Therefore, they can be used as highly effective, reversible and safe contraceptives for both male and female. They can be also utilized for treatment of various diseases related to disorders of reproductive endocrine such as hormonedependent prostate cancer and breast cancer, endometriosis, precosious puberty of children. They are also useful in treatment of infertility. The new LHRH antagonists herein can also be utilized in the basic research of reproductive physiology and pharmacology, such as in the study on the function of piturtary gland, on the effect of gonadal hormones or gonadotrpins or LHRH on sexual behaviour, etc.

ABBREVIATIONS

The following are abbreviations which have been used in the text of this patent application document.

Ala	alanine
AOA	antiovulatory activity
Arg	arginine
Bap	dibutylaminomethyl phenylalanine
Boc	t-butyloxylcarbonyl
BuOAC	butyl acetate
CAT	cutaneous anaphalactoid test
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
D2Nal	D-β-(2-naphthyl) alanine
D3Pal	D-β-(3-pyridyl) alanine
DpClPhe	p-chloro-D-phenylalanine
DpFPhe	p-fluoro-D-phenylalanine
D6Qal	D-β-(6-quinolyl) alanine
DMF	N,N-dimethyl formamide
Eap	diethylaminomethyl phenylalanine
ED_{50}	effective dose for 50 % response
EtOAC	ethyl acetate
FSH	follicle-stimulating hormone
Glu	glutamic acid
Gly	glycine
His	histidine
HOBT	1-hydroxybenzoltriazole
HPLC	high performance liquid chromatography
	ninhydrin derivation
HRA	histamine-releasing activity
HRT	histamine-releasing test
IEN	ion exchange chromatography with post-column

IPA	isopropyl alcohol
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
Leu	leucine
Lys	lysine
Map	dimethylaminomethyl phenylalanine
Met	methionine
Mop	mophorlinomethyl phenylalanine
nBuOH	n-butyl alcohol
NS	normal saline
Pap	dipropylaminomethyl phenylalanine
Phe	phenylalanine
Pip	piperidinomethyl phenylalanine
Pipes	piperazine-N,N'-bis[2-ethanesulfonic acid]
Pro	proline
Rf	rate of flow
SE	standard error
Ser	serine
TFA	trifluoracetic acid
TLC	thin-layer chromatography
TR	retention time
Trp	tryptophan
Tyr	tyrosine
Tep	tetrahydroperrolyl methyl phenylalanine

Claims

1. A method for designing and synthesizing LHRH antagonists by taking highly potent LHRH antagonist [NAc-D2Nal^1, DpClPhe^2, D3Pal^3, Ser^4, Tyr^5, Arg^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2 (II) as parent compound and modifying both alkalinous and lipophilic areas of the molecule of (II), to obtain new LHRH antagonists having both high antiovulatory activity (AOA) and low histaminereleasing activity (HRA) based on its topological similarity with the molecule of a niuropeptide, Substance P.

2. The method and process of design and synthesis based on claim 1 wherein Tyr^5-DArg^6-Arg^8 in C-terminus and aromaric amino acids in N-terminus in (II) is adjusted and replaced.

3. The method of design and synthesis based on claim 1 and 2 wherein suitable alkalinous group is introduced into position 2,3,5,6,8 and unnatural amino acid is inserted in the above mentioned positions.

4. The method of design and synthesis based on claim 1 wherin D3Pal having suitable basicity is substituted for DArg^6 in (II) to give analog (III): [NAc-D2Nal^1, DpClPHe^2, D3Pal^3, Ser^4, Tyr^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2

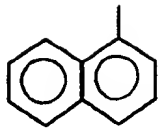
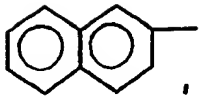
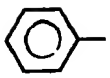
5. The method of design and synthesis based on claim 1 and 4 wherein Arg^5 is substitued for Tyr^5 in (III) to give (IV) [NAc-D2Nal^1, DpClPhe^2, D3Pal^3, Ser^4, Arg^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2

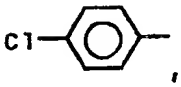
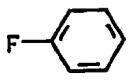
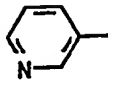
6. The method and process of design and synthesis based on claim 5 wherein DPhe^3 is substituted for D3Pal^3 in (IV) to give (V): [Nac-D2Nal^1, DpClPhe^2, Dphe^3, Ser^4, Arg^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2

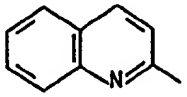
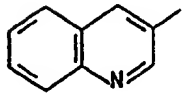
7. The method and process of design and synthesis based on claim 4 wherin DPhe^3 is substituted for D3Pal^3 in (III) to give (V'): [NAc-D2Nal^1, DpClPhe^2, DPhe^3, Ser^4, Tyr^5, D3Pal^6, Leu^7, Arg^8, Pro^9, DAla^{10}]NH_2

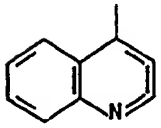
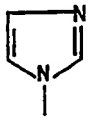
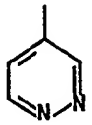
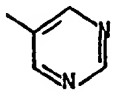
8. A compound as described in claim 1 which is expressed as the formula, [NAc-D2Nal^1, AA^2, AA^3, Ser^4, AA^5, AA^6, Leu^7, AA^8, Pro^9, DAla^{10}]-NH_2, in which AA are natural or unnatural amino acids on the formula D-or L-ArAla

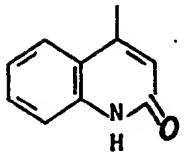
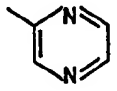
wherein

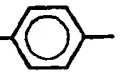
Ar =  ,  , H ,  ,

 ,  ,  ,  ,

 ,  ,  ,

 ,  ,  ,  ,

 ,  ,

and XCH_2  ,

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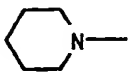
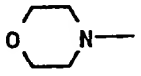
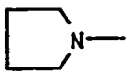
X=  ,  ,  ;

and R_2^1N-, RR'N-:

in which

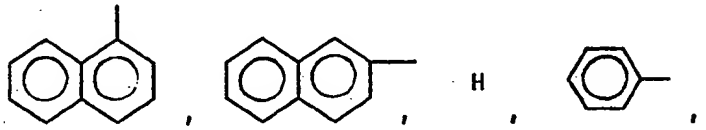
R' = CH_3-, CH_3CH_2-, C_3H_7-, C_4H_9-, H-;

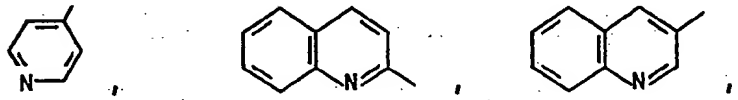
R = CH_3-, CH_3CH_2-, C_3H_7-, C_4H_9-, H-;

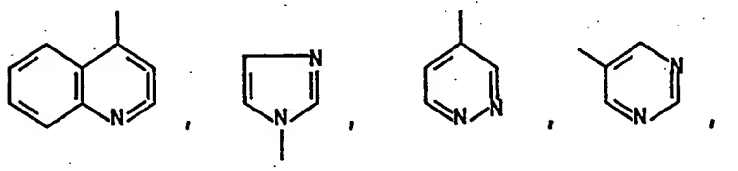
9. The LHRH antagonists based on claim 8

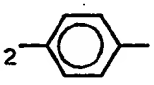
AA^2 = D-pClPhe, D-ArAla, DPhe, Ar-Ala, $DXCH_2Phe$;
AA^3 = D3Pal, Ar-Ala, D-ArAla, DPhe, $D\text{-}XCH_2Phe$;
AA^5 = Arg, DMap, Pip, Tyr, Pal, Mop, Tep, Map, Phe, Eap, Pap, Bap, DMop;
AA_6 = D3Pal, D-Ar-Ala, $D\text{-}XCH_2Phe$;
AA^8 = Pip, Mop, Tep, Map, Eap, Pap, Bap, Arg;

in which

Ar = 





and XCH_2 

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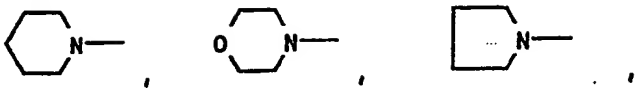
X= 

and R'_2N-, RR'N-

in which

R' = CH_3-, CH_3CH_2-, C_3H_7-, C_4H_9-, H-;

R = CH_3-, CH_3CH_2-, C_3H_7-, C_4H_9-, H-;

10. The application of LHRH antagonists as claimed in claim 8 odr 9 wherein the compound, as peptide medicine formulated as normal injection, injectable capsules and other pharmaceutical compositions is used for treating disorder in reproductive endocrinology system, including endometriosis, precocious puberty of children, prostate cancer and breast cancer, and for birth control as male or female contraceptive medicine or used for diagnosing and treating infertility.

11. [N-AC-D2Nal^1, P-Cl-D-Phe^2, D3Pal^3, Ser^4, Mop^5, D3Pal^6, Leu^7, Arg^8, Pro^9, D-Ala^{10}]NH_2

12. [N-AC-D-2Nal^1, D-Phe^2, D3Pal^3, Ser^4, Mop^5, D3Pal^6, Leu^7, Arg^8, Pro^9, D-Ala^{10}]NH_2

13. [N-AC-D-2Nal^1, P-Cl-D-Phe^2, D3Pal^3, Ser^4, Arg^5, D3Pal^6, Leu^7, Pap^8, Pro^9, D-Ala^{10}]NH_2

Figure 1: The TLC result of LHRH antagonists IV, V, VII in five different systems

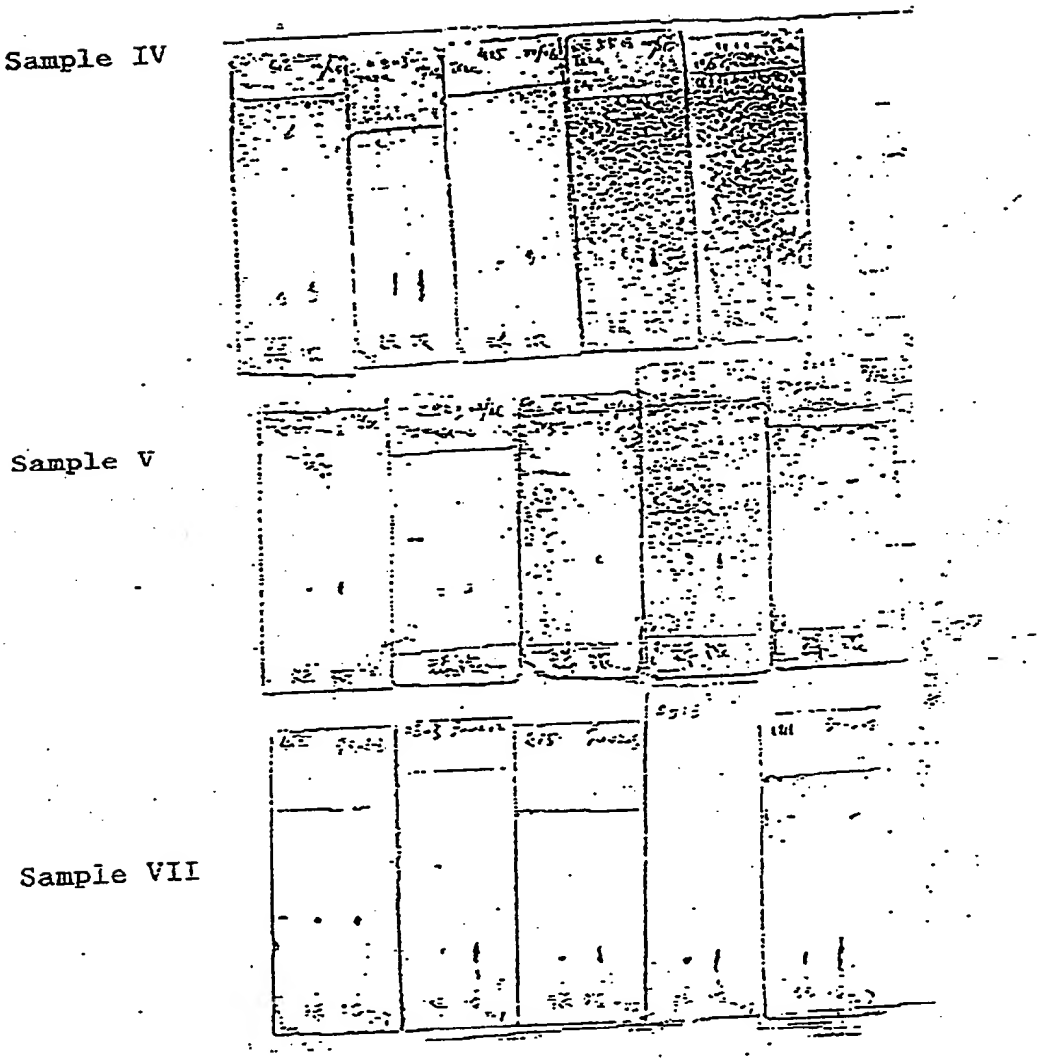


Figure 2: The reversed phase HPLC spectra for the pure sample of LHRH antagonist IV

Conditions:

Column: μ-Bondapak C18 (3.9 mm X cm)
moble phase: A, 0,1 M NH_4OAC (pH7)
B, 20 % A + 80 % acetonitrile
gradient procedure: B from 10 % to 100 % in 15 minutes
flow rate: 2 ml/minute
detector: UV 229 nm

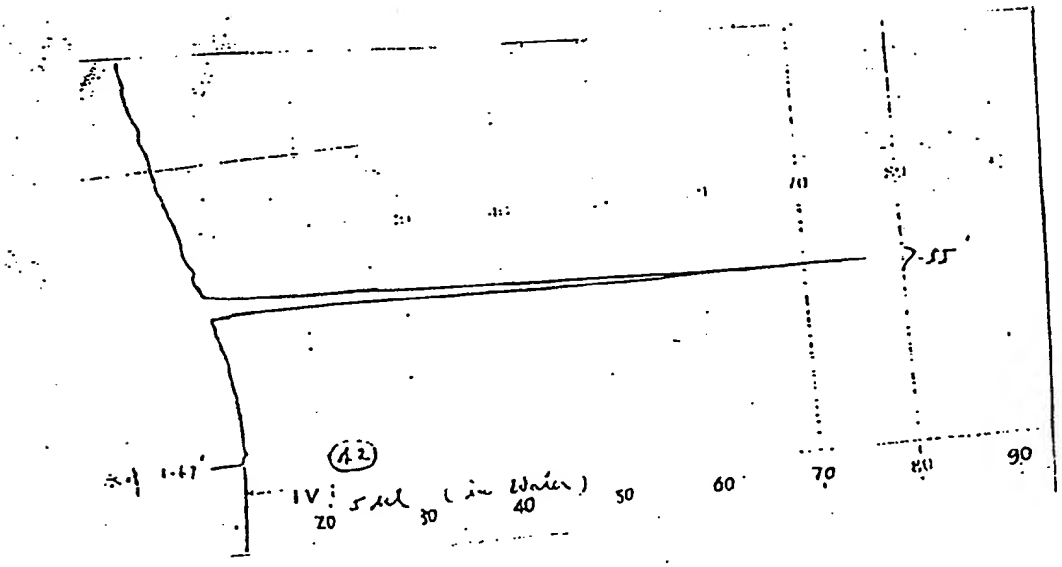


Figure 3: The HPLC spectra for the pure sample of LHRH antagonist V

Conditions:

Column: μ-Bondapak C18 (3,9 mm X 30 cm)
moving phase: A, 0.01 M KH_2PO_4 pH 3)
B, 20 % A + 80 % acetonitrile
gradient procedure: B from 40 % to 100 % in 15 minutes
flow rate: 2 ml/minute
detector: UV 210 nm

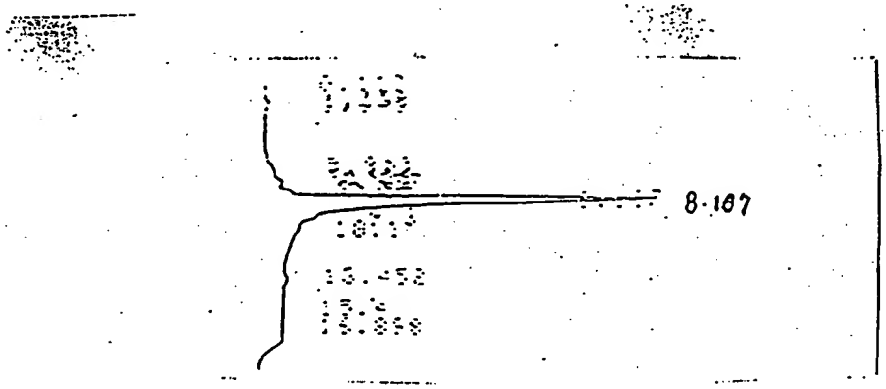


Figure 4: The HPLC spectra for the pure sample of LHRH antagonist VII

Conditions:

Column: μ-Bondapak C18 (3,9 mm X 30 cm)
moving phase: A, 0,01 M KH_2PO_4 (pH 3)
B, 20 % A + 80 % acetonitrile
gradient procedure: B from 40 % to 100 % in 15 minutes
flow rate: 1 ml/minute
detector: UV 210 nm

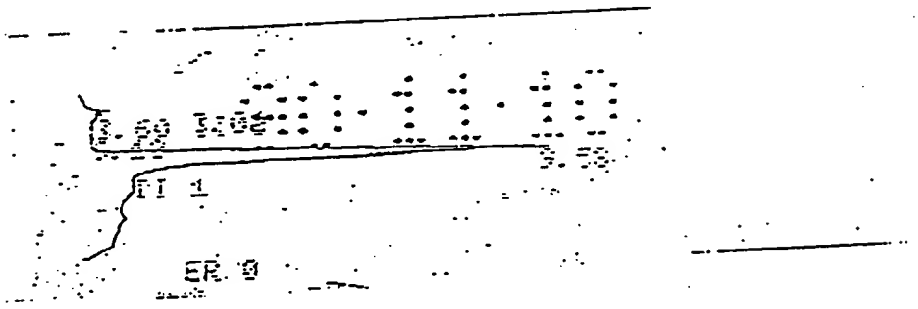


Figure 5: The PICO-TAGTM spectra of LHRH antagonist IV

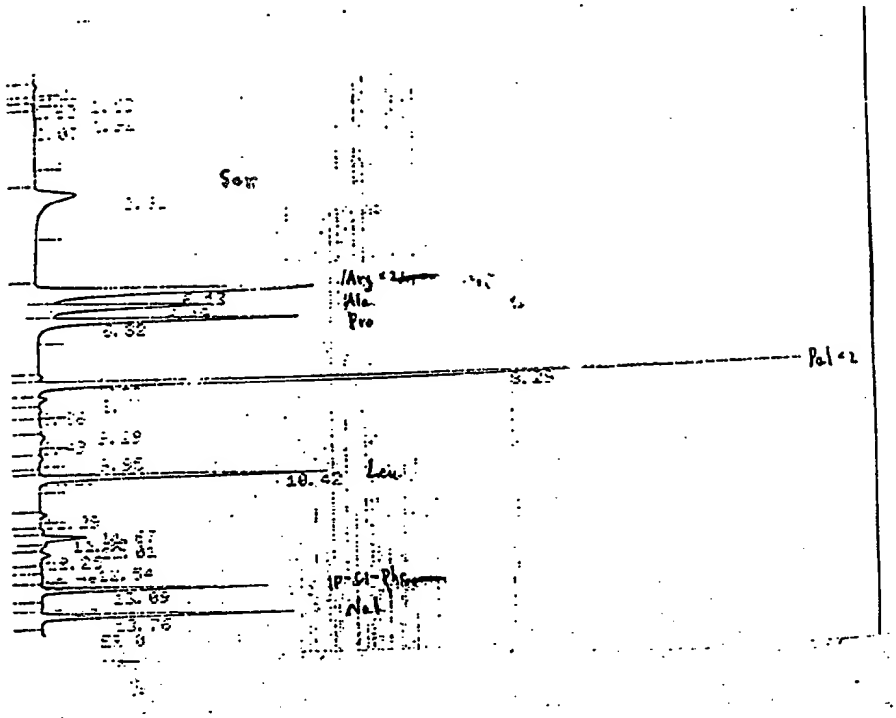
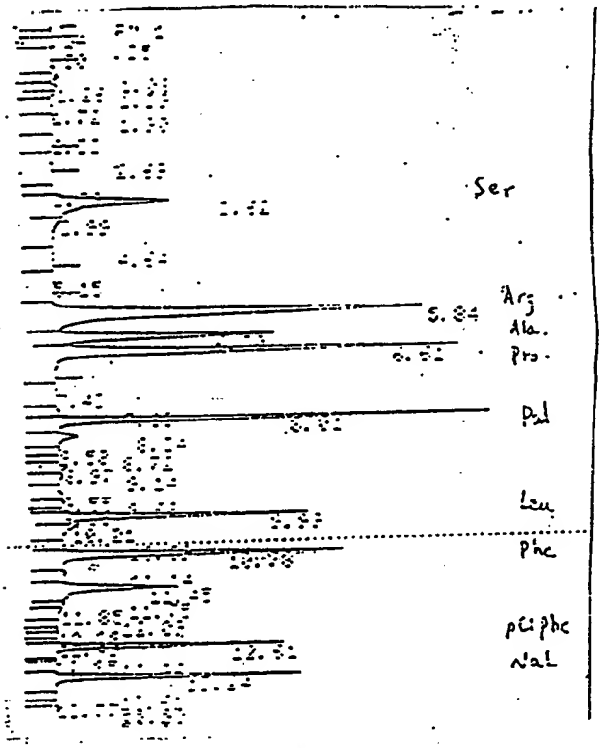


Figure 6: The PICO-TAGTM spectra of LHRH antagonist V



International Appli No PCT/EP 91/02110

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 7/20 A 61 K 37/43

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	International Journal of Peptide & Protein Research, vol. 35, no. 2, February 1990, Copenhagen (DK), K. Liu et al.: "Antagonists of luteinizing hormone releasing hormone with novel unnatural amino acids at position six", pages 157-160, see the whole article ---	1-5,8-10
Y	---	1-3,6-13
X	Endocrine Reviews, vol. 7, no. 1, The Endocrine Society, M.J. Karten et al.: "Gonadotropin-releasing hormone analog design. Structure-function studies toward the development of agonists and antagonists: rationale and perspective", pages 44-66, see especially page 59, column 2 - page 60, column 2 --- -/-	1-3

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
31-01-1992	03. 02. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme. M. van der Drift

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	---	1-13
X	Z. Naturforsch., vol. 41C, 1986, Tübingen (DE), K. Folkers et al.: "Relative potencies of antagonists of the luteinizing hormone releasing hormone with Lys8 and Arg8 and substitutions in positions 3,5,6 and 8", pages 1087-1091, see the whole article	1-5,8-10
Y	---	1-3,6-13
X	Z. Naturforsch., vol. 42B, 1987, Tübingen (DE), K. Folkers et al.: "Activities of antagonists of the luteinizing hormone releasing hormone with emphasis on positions 1,5 and 6 and on postions 1,2 and 3", pages 101-106, see the whole article	1-4,8-10
Y	---	1-3,5-13
X	B.H. Vickery and J.J. Nestor Jr, MTP Press, Boston (US), 1987, R.W. Roeske et al.: "LHRH antagonists with low histamine releasing activity", pages 17-24, see page 19	1-5,8-10
Y	---	1-3,6-13
X	EP,A,0277829 (SYNTEX (USA) INC.) 10 August 1988, see tables 1,3,4; claims 1-25	1-3
Y	---	1-13
P,X	Science in China, vol. 34, no. 2, February 1991, series B, K.L. Liu et al.: "Synthesis and bioactivities of new LHRH antagonists containing novel unnatural amino acids at position five", pages 201-208, see the whole article	1-3,8-12

FURTHER INFORMATION C TINUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers 10 because they relate to subject matter not required to be searched by this Authority, namely:

" REMARK: Although claim 10 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition."

2. [ ] Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this International application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9102110 SA 52751

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/02/92 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0277829	10-08-88	US-A- 4801577	31-01-89
		AU-B- 614275	29-08-91
		AU-A- 1126588	11-08-88
		JP-A- 63201199	19-08-88